EXHIBIT 170

Original Research

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PTI-125 Reduces Biomarkers of Alzheimer's Disease in Patients

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Abstract

BACKGROUND: The most common dementia worldwide, Alzheimer's disease is often diagnosed via biomarkers in cerebrospinal fluid, including reduced levels of Aβ1–42, and increases in total tau and phosphorylated tau-181. Here we describe results of a Phase 2a study of a promising new drug candidate that significantly reversed all measured biomarkers of Alzheimer's disease, neurodegeneration and neuroinflammation. PTI-125 is an oral small molecule drug candidate that binds and reverses an altered conformation of the scaffolding protein filamin A found in Alzheimer's disease brain. Altered filamin A links to the α7-nicotinic acetylcholine receptor to allow Aβ42's toxic signaling through this receptor to hyperphosphorylate tau. Altered filamin A also links to toll-like receptor 4 to enable Aβ-induced persistent activation of this receptor and inflammatory cytokine release. Restoring the native shape of filamin A prevents or reverses filamin A's linkages to the α7-nicotinic acetylcholine receptor and toll-like receptor 4, thereby blocking Aβ42's activation of these receptors. The result is reduced tau hyperphosphorylation and neuroinflammation, with multiple functional improvements demonstrated in transgenic mice and postmortem Alzheimer's disease brain.

OBJECTIVES: Safety, pharmacokinetics, and cerebrospinal fluid and plasma biomarkers were assessed following treatment with PTI-125 for 28 days. Target engagement and mechanism of action were assessed in patient lymphocytes by measuring 1) the reversal of filamin A's altered conformation, 2) linkages of filamin A with α7-nicotinic acetylcholine receptor or toll-like receptor 4, and 3) levels of Aβ42 bound to α7-nicotinic acetylcholine receptor or CD14, the co-receptor for toll-like receptor 4.

DESIGN: This was a first-in-patient, open-label Phase 2a safety, pharmacokinetics and biomarker study.

SETTING: Five clinical trial sites in the U.S. under an Investigational New Drug application.

PARTICIPANTS: This study included 13 mild-to-moderate Alzheimer's disease patients, age 50-85, Mini Mental State Exam ≥16 and ≤24 with a cerebrospinal fluid total tau/Aβ42 ratio ≥0.30.

INTERVENTION: PTI-125 oral tablets (100 mg) were administered twice daily for 28 consecutive days.

MEASUREMENTS: Safety was assessed by electrocardiograms, clinical laboratory analyses and adverse event monitoring. Plasma levels of PTI-125 were measured in blood samples taken over 12 h after the first and last doses; cerebrospinal fluid levels were measured after the last dose. Commercial enzyme linked immunosorbent assays assessed levels of biomarkers of Alzheimer's disease in cerebrospinal fluid and plasma before and after treatment with PTI-125. The study measured biomarkers of pathology (pT181 tau, total tau and Aβ42), neurodegeneration (neurofilament light chain and neurogranin) and neuroinflammation (YKL-40, interleukin-6, interleukin-1β and tumor necrosis factor α). Plasma levels of phosphorylated and nitrated tau were assessed by immunoprecipitation of tau followed by immunoblotting of three different phospho-epitopes elevated in AD (pT181-tau, pS202-tau and pT231-tau) and nY29-tau. Changes in conformation of filamin A in lymphocytes were measured by isoelectric focusing point. Filamin A linkages to α7-nicotinic acetylcholine receptor and toll-like receptor 4 were assessed by immunoblot detection of α7-nicotinic acetylcholine receptor and toll-like receptor 4 in anti-filamin A immunoprecipitates from lymphocytes. Aβ42 complexed with α7-nicotinic acetylcholine receptor or CD14 in lymphocytes was also measured by co-immunoprecipitation. The trial did not measure cognition.

RESULTS: Consistent with the drug's mechanism of action and preclinical data, PTI-125 reduced cerebrospinal fluid biomarkers of Alzheimer's disease pathology, neurodegeneration and neuroinflammation from baseline to Day 28. All patients showed a biomarker response to PTI-125. Total tau, neurogranin, and neurofilament light chain decreased by 20%, 32% and 22%, respectively. Phospho-tau (pT181) decreased 34%, evidence that PTI-125 suppresses tau hyperphosphorylation induced by Aβ42's signaling through α7-nicotinic acetylcholine receptor. Cerebrospinal fluid biomarkers of neuroinflammation (YKL-40 and inflammatory cytokines) decreased by 5-14%. Biomarker effects were similar in plasma. Aβ42 increased slightly – a desirable result because low Aβ42 indicates Alzheimer's disease. This increase is consistent with PTI-125's 1,000-fold reduction of Aβ42's femtomolar binding affinity to α7-nicotinic acetylcholine receptor. Biomarker reductions were at least $p \leq 0.001$ by paired t test. Target engagement was shown in lymphocytes by a shift in filamin A's conformation from aberrant to native: 93% was aberrant on Day 1 vs. 40% on Day 28. As a result, filamin A linkages with α7-nicotinic acetylcholine receptor and toll-like receptor 4, and Aβ42 complexes with α7-nicotinic acetylcholine receptor and CD14, were all significantly reduced by PTI-125. PTI-125 was safe and well-tolerated in all patients. Plasma half-life was 4.5 h and approximately 30% drug accumulation was observed on Day 28 vs. Day 1.

CONCLUSIONS: PTI-125 significantly reduced biomarkers of Alzheimer's disease pathology, neurodegeneration, and neuroinflammation in both cerebrospinal fluid and plasma.

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JPAD - Volume 7, Number 4, 2020

All patients responded to treatment. The magnitude and consistency of reductions in established, objective biomarkers imply that PTI-125 treatment counteracted disease processes and reduced the rate of neurodegeneration. Based on encouraging biomarker data and safety profile, approximately 60 patients with mild-to-moderate AD are currently being enrolled in a Phase 2b randomized, placebo-controlled confirmatory study to assess the safety, tolerability and efficacy of PTI-125.

Key words: tau, neurofilament light chain, neurogranin, YKL40, neuroinflammation.

Introduction

Alzheimer's disease (AD) is the most common dementia, afflicting fifty million people worldwide (1). PTI-125 is a new small molecule drug candidate for AD with a novel mechanism of action: it binds and restores to normal an altered conformation of the scaffolding protein filamin A (FLNA) (2-4). PTI-125's target, an altered conformation of FLNA, is a known proteopathy in AD brain (2, 3). FLNA is a large, 280-KDa intracellular scaffolding protein best known for cross-linking actin to regulate cell structure and motility and is highly expressed in brain. FLNA dimerizes by a domain in its membrane-bound C terminal, protruding into the cytoplasm as an inverse V shape to interact with at least 90 different proteins (5), indicating potential involvement in numerous intracellular processes. In the cell membrane, FLNA constitutively associates with certain receptors such as the insulin receptor and the mu opioid receptor, and FLNA regulates insulin receptor signaling and mu opioid receptor – G-protein coupling (6). FLNA does not normally link to α7-nicotinic acetylcholine receptor (α7nAChR) or toll-like receptor 4 (TLR4); however, in AD brain, the altered conformation of FLNA enables FLNA's association with both (3). Importantly, the aberrant FLNA linkages of altered FLNA to α7nAChR and TLR4 promote toxic signaling of soluble Aβ42 via each receptor, contributing to AD pathology (3, 4).

The extremely tight binding (high femtomolar affinity) of Aβ42 to α7nAChR, first demonstrated two decades ago (7), is reinforced by altered FLNA's linkage to this receptor (3). Aβ42 signals via α7nAChR to hyperphosphorylate tau protein by activating kinases ERK and JNK1 (8). Tau hyperphosphorylation is a hallmark of AD pathology and disrupts tau's function of stabilizing microtubules, promoting degeneration. Hyperphosphorylated tau also initiates neurofibrillary lesions and tau protofibril formation, leading to eventual fibrillar tau-rich tangles. This toxic signaling pathway of Aβ42 via α7nAChR has been confirmed by multiple laboratories under conditions that maintain Aβ42 as soluble monomers or small oligomers (9-11).

Aβ42 activates TLR4 by binding the TLR4 co-receptor CD14 (12). Aβ42 binding to CD14 promotes a sustained activation of TLR4 and persistent release of inflammatory cytokines such as IL-6, IL-1β and TNFα (13). Like Aβ42 signaling through α7nAChR, this Aβ42-induced TLR4 activation requires the linkage of altered FLNA to TLR4 (4). The chronic activation of TLR4 by amyloid in AD leads to neuroinflammation and exacerbates neurodegeneration.

By preferentially binding the altered conformation of FLNA and restoring its native shape, PTI-125 releases FLNA from α7nAChR and TLR4, reducing Aβ42-driven tau hyperphosphorylation and neuroinflammation, thereby attenuating neurodegeneration (2). In triple transgenic AD mice, PTI-125 restored α7nAChR, NMDAR and insulin receptor signaling, improved synaptic plasticity, reduced amyloid deposits and neurofibrillary lesions, robustly attenuated inflammatory cytokine levels, and improved cognition (3). In vitro PTI-125 incubation of postmortem human AD brain (or age-matched control brain treated with exogenous Aβ42) also reduced FLNA linked to α7nAChR or TLR4, decreased Aβ42 – α7nAChR complex levels, decreased Aβ42-induced tau hyperphosphorylation, and again improved synaptic plasticity and receptor function. IC50s for these effects were nanomolar, and significant effects were seen at concentrations as low as 1 picomolar (3, 4).

This Phase 2a clinical trial follows favorable safety and tolerability data from a Phase 1 study of 50, 100, or 200 mg of PTI-125 in healthy volunteers. In this first-in-patient, Phase 2a clinical trial, thirteen mild-to-moderate AD patients received 100 mg oral PTI-125 b.i.d. for 28 days. Patients were age 50-85, MMSE ≥ 16 and ≤ 24, with a CSF T-tau/Aβ42 ratio ≥ 0.30. The 100 mg dose was selected because it is equivalent by body surface area conversion (the accepted method of determining equivalent doses between species) to effective daily doses of PTI-125 in AD mouse models (3, 4). The T-tau/Aβ42 ratio was selected as a low cutoff to confirm AD diagnosis based on biomarker determinations from samples obtained from the Swedish bioFINDER and ADNI (Alzheimer's Disease Neuroimaging Initiative) cohorts (14), recognizing that we used commercial enzyme-linked immunosorbent assays (ELISAs) and that study used ElectroChemiLuminescence Immunoassays (ECLIAs). Although these assays are not directly comparable, values from each are reported in pg/mL. Safety was monitored by electrocardiograms, clinical labs, adverse event monitoring and physical examinations. Change from baseline was measured for CSF and plasma biomarkers of AD pathology (T-tau, P-tau and Aβ42), neurodegeneration (neurofilament light (NfL) chain and neurogranin) and neuroinflammation (YKL-40 and inflammatory cytokines IL-6, IL-1β and TNFα). Pre-dose and Day 28 samples were tested in triplicate in a single ELISA plate according to manufacturers' instructions.

A recent meta-analysis showed that in CSF of AD

Table 1. ELISA kits used to measure CSF and plasma biomarkers

Biomarker	Manufacturer	Catalog #	Type
Total tau	Invitrogen Thermo	KHB0041	Sandwich
pT181-tau	Invitrogen Thermo	KHO0631	Sandwich
Aβ42	Invitrogen Thermo	KHB3441	Sandwich
Neurofilament light chain	Lifespan	LS-F6701	Sandwich
Neurogranin	Lifespan	LS-F6894	Indirect
YKL-40	Lifespan	LS-F1020	Sandwich
IL-6	Lifespan	LS-F9982	Sandwich
IL-1β	Lifespan	LS-F5625	Sandwich
TNFα	Invitrogen Thermo	KHC3011	Sandwich

patients vs. age-matched controls, T-tau and P-tau are increased respectively by 2.5- and 1.9-fold and Aβ42 is reduced by half (15). NfL, expressed predominantly in large caliber axons and indicating axonal damage, is increased by 2.3-fold in CSF of AD patients relative to controls (15). NfL may track disease progression and is emerging as a plasma AD biomarker (16). Neurogranin, a post-synaptic protein in dendritic spines, is elevated in AD and represents synaptic/dendritic destruction (17-19). The secreted glycoprotein YKL-40, an inflammation mediator, is thought to accompany microglial activation and extracellular tissue remodeling and is also elevated in AD (18, 20, 21).

Methods

Patients

Of 19 patients screened, 13 enrolled. All 13 were mild-to-moderate AD patients, age 50-85, MMSE ≥ 16 and ≤ 24, with a CSF T-tau/Aβ42 ratio ≥ 0.30. Although the protocol stated 12 patients would be enrolled, 13 were enrolled due to multiple patients in screening near the end of the study.

Lymphocyte and plasma preparation

To prepare lymphocytes, 8 ml venous blood collected in EDTA-containing tubes was layered onto 8 ml Histopaque-1077 at 25°C and centrifuged (400 g, 30 min, 25°C) to yield plasma (top layer) and lymphocytes (opaque interface). Plasma fractions were aliquoted into Eppendorf centrifuge tubes and stored at -80°C until assay. The obtained lymphocytes were washed twice by mixing with 10 ml phosphate-buffered saline (PBS) followed by centrifugation at 250 g for 10 min. The final lymphocyte pellet was resuspended in 600 μl cell freezing medium (DMEM, 5% DMSO, 10% fetal bovine serum), aliquoted and held at -80°C until assay.

Treatment of CSF and plasma for biomarkers

CSF and plasma were thawed on ice and immediately treated with 20X protease inhibitor cocktail (Complete mini EDTA-free protease inhibitors, Roche, 04693159001) and protein phosphatase inhibitor cocktail (Phosphostop phosphatase inhibitors, Roche, 04906837001).

Determination of CSF and plasma biomarkers

Levels of biomarkers in protease and protein phosphatase inhibitor treated CSF and plasma were measured by enzyme-linked immunosorbent assays (ELISA) in triplicate against standards, according to manufacturer's instruction. The ELISA kits (Table 1) from Invitrogen or Lifespan were solid phase sandwich ELISAs (except neurogranin, a solid phase indirect ELISA) that detect endogenous levels of biomarkers with a specific detecting antibody followed by an anti-species IgG, horseradish peroxidase (HRP)-linked antibody to recognize the bound detection antibody. HRP substrate tetramethylbenzidine was added to develop color. Absorbance for the developed color is proportional to the quantity of protein. Absorbances were analyzed against standards by linear regression using GraphPad Prism.

Measurement of FLNA – α7nAChR/TLR4 linkages and Aβ42 –α7nAChR/CD14 complexes in lymphocytes and ex vivo Aβ42 treatment

Levels of FLNA – α7nAChR/TLR4 and Aβ42 – α7nAChR/CD14 interactions were assessed in patient lymphocytes. Lymphocytes (200 μg) from patients at the indicated dosing days were incubated at 37°C with oxygenated protease inhibitors containing Kreb's Ringer (K-R) or 0.1 μM Aβ42 for 30 min (250 μl total incubation volume). The assay mixtures were aerated with 95%O2/5%CO2 for 1 min every 10 min. Reactions were terminated by adding ice-cold Ca2+-free K-R containing protease and protein phosphatase

JPAD - Volume 7, Number 4, 2020

inhibitors (Roche) and centrifuging. The obtained lymphocytes were homogenized in 250 µl ice-cold immunoprecipitation buffer (25 mM HEPES, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.2% 2-mercaptoethanol with protease and protein phosphatase inhibitors) by sonication for 10 s on ice, and solubilized by nonionic detergents (0.5% NP-40/0.2% Na cholate/0.5% digitonin) for 60 min (4°C) with end-to-end rotation. The obtained lysate was centrifuged at 20,000 g for 30 min (4°C) and the resultant supernatant (0.25 ml) was diluted 4x with 0.75 ml immunoprecipitation buffer. Aβ42 – α7nAChR complexes were immunoprecipitated with immobilized anti-FLNA (SC-58764 + SC-17749, Santa Cruz) or anti-Aβ42 (AB5078P, Millipore Sigma) + anti-actin (SC-8432+ SC-376421) antibodies onto protein A/G-conjugated agarose beads (#20421, Thermo). Resultant immunocomplexes were pelleted by centrifugation (4°C), washed 3x with ice-cold PBS, pH 7.2, containing 0.1% NP-40, and centrifuged. Immunocomplexes were solubilized by boiling 5 min in 100 µl SDS-PAGE sample preparation buffer (62.5 mM Tris-HCl, pH 6.8; 10% glycerol, 2% SDS; 5% 2mercaptoethanol, 0.1% bromophenol blue) and centrifuged to remove antibody-protein A/G agarose beads. Levels of α7nAChRs (SC-58607) and β-actin (SC-47778) were determined by immunoblotting, with FLNA and β-actin levels serving as the indicators of immunoprecipitation efficiency and gel loading (3, 4, 22). Blots were then stripped and re-probed with specific antibodies against TLR4 (SC-302972) and CD14 (SC-1182) to assess levels of FLNA – TLR4 and Aβ42 – CD14 associations, respectively. Blots of anti-Aβ42 immunoprecipitates size-fractionated on 20% SDS-PAGE were used to survey the molecular mass of Aβ42 using anti-Aβ42 (AB5078P, Millipore Sigma).

Measurement of phosphorylated and nitrated tau in plasma

Phosphorylated and nitrated tau in plasma were assessed using an established method (3, 8, 23). Total tau proteins were immunoprecipitated by 1 µg immobilized anti-tau (SC-65865 and SC-166060), which does not discriminate between phosphorylation states. Levels of phosphorylated tau (pS202tau, pT231tau and pT181tau), nitrated tau (nY29tau) and total tau precipitated (loading control) were assessed by immunoblotting with antibodies specific to each phosphoepitope (pS202tau: AT8 [MN1020], pT231tau: AT180 [MN1040] and pT181tau: AT270 [MN1050], all from Thermo Invitrogen), anti-nY29tau (SC-66177), and anti-tau (SC-32274).

Isoelectric point assessment

Lymphocyte FLNA was isolated using a procedure established for brain tissues (3) with slight modification. Lymphocytes (200 µg) were sonicated for 10 s on ice in 200 µl modified hypotonic solution (50 mM Tris HCl, pH 8.0, 11.8 mM NaCl, 0.48 mM KCl, 0.13 mM CaCl2, 0.13 mM Mg2SO4, 2.5 mM NaHCO3) with a cocktail of protease and protein phosphatase inhibitors. The treated lymphocytes were solubilized using 0.5% digitonin/0.2% sodium cholate/0.5% NP-40 (4°C) with end-over-end rotation for 1 h. Following centrifugation to remove insoluble debris, the lysate was treated with 1% sodium dodecyl sulfate (SDS) for 1 min to dissociate FLNA-associated proteins, diluted 10x with immunoprecipitation buffer, and immunopurified with immobilized anti-FLNA (SC-58764 + SC-17749). Resultant FLNA was eluted using 200 µl antigen-elution buffer (#21004, Thermo), neutralized immediately with 100 mM Tris HCl (pH 9.0), diluted to 500 µl with 50 mM Tris HCl, pH 7.5, and passed through a 100-kD cut-off filter to remove low-molecular weight FLNA fragments. Once purified, FLNA was suspended in 100 µl isoelectric focusing sample buffer. Samples (50 µl) were loaded onto pH 3-10 isoelectric focusing gels and proteins fractionated (100 V for 1 h, 200 V for 1 h, and 500 V for 30 min). Separated proteins were then electrophoretically transferred to nitrocellulose membranes. FLNA was identified by immunoblotting with anti-FLNA (SC-57864) antibodies.

Pharmacokinetic methods

Blood samples (4 mL) were drawn into a Vacutainer® tube containing K_2EDTA, placed on ice, and centrifuged 1000 xg for 15 min. Plasma was split into two aliquots, transferred to polypropylene tubes and stored at -20°C or below until analysis.

Plasma PK parameters for PTI-125 were calculated using non-compartmental methods in WinNonlin. The peak drug concentration (C_{max}), the time to peak drug concentration (T_{max}), T_{last} and C_{last}, the time to the last quantifiable drug concentration, were obtained directly from the data without interpolation. The following parameters were calculated: the elimination rate constant (λ_z), the terminal elimination half-life ($T_{1/2}$), the AUC from time zero to the time of the last quantifiable concentration (AUC_{last}), the AUC from time zero extrapolated to infinity (AUC_{inf}), and the percentage of AUC_{inf} based on extrapolation (AUC_{extrap}(%)), Cl/F, the apparent oral clearance, and Vz/F, apparent volume of distribution. Accumulation was estimated by the ratios of the AUC on the last day of dosing to the corresponding AUC the first day of dosing.

Below limit of quantitation (BLQ) concentrations were treated as zero from time-zero up to the time at which the first quantifiable concentration was observed; embedded and/or terminal BLQ concentrations were treated as "missing." Full precision concentration data and actual sample times were used for all pharmacokinetic and statistical analyses.

Table 2. Mean PK parameters of PTI-125 100 mg b.i.d. in AD patients (± SD)

Day	Cmax (ng/mL)	Tmax (h)	Clast (ng/mL)	Tlast (h)	λz (1/h)	AUClast (h*ng/mL)	T1/2 (h)	CSF/plasma ratio
Day 1	1020 ± 442	2.00 (1.00-3.00)	176 ± 112	12 ± 0.015	0.176 ± 0.496	5320 ± 2230	4.51 ± 2.43	---
Day 28	1100 ± 417	2.06 (1.00-5.93)	238 ± 168	12 ± 0.029	0.174 ± 0.051	6700 ± 3240	4.35 ± 1.39	0.61 ± 0.41

Note: Tmax is reported as median (min-max)

Statistics and blinding

All CSF and plasma ELISA biomarker data were analyzed by two-sided paired t test by an independent statistician. Plasma tau phosphorylation and lymphocyte data including FLNA conformation were analyzed by one-way ANOVA with post-hoc two-sided t test (unpaired) on all 13 patients, with one missing (baseline) value. All biomarker assessments were performed blind to treatment day; samples were coded prior to testing.

Results

Demographics

This first-in-patient Phase 2a trial enrolled 13 patients: 9 females, 4 males; 3 black, 10 white; 6 Hispanic, 7 non-Hispanic. Both CSF and plasma/lymphocyte data were n=12 (or n=13 with one missing value) because one patient declined the second CSF draw, and the baseline plasma/lymphocyte sample was missing for another patient. Additionally, one patient stopped dosing on Day 21, was tested positive for cocaine on that day, did not resume dosing, but returned for the CSF draw and whole blood sample on Day 28. This patient's CSF and plasma biomarker data are included.

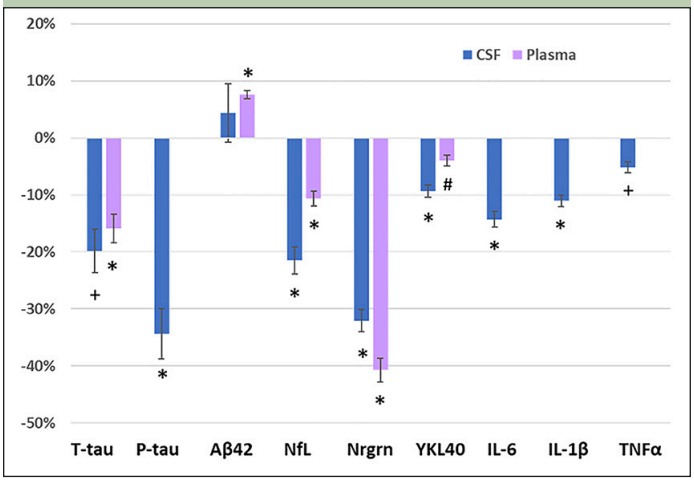
Safety and pharmacokinetics

In this Phase 2a trial, PTI-125 was safe and well tolerated with no drug-related adverse events. Half-life was 4.5 h, and approximately 30% accumulation was observed on Day 28 compared to Day 1 exposure. PK parameters are shown in Table 2. The CSF to plasma ratio of the PTI-125 analyte used plasma levels from the nearest PK time point to the time of CSF draw on Day 28, or the average of two if in the middle. Ratios ranged from 0.09 to 1.2, with CSF draw times ranging from 1.15 to 7.75 h post-dose, with higher ratios trending to later time points.

CSF and plasma biomarkers

Consistent with PTI-125's mechanism of action and preclinical data, PTI-125 treatment reduced CSF biomarkers of neurodegeneration and AD pathology from baseline to Day 28 (Fig. 1). T-tau decreased 20%, neurogranin decreased 32%, and NfL decreased 22%. P-tau (pT181) decreased 34%, evidence that PTI-125 suppresses tau hyperphosphorylation induced by Aβ42's signaling through α7nAChR. CSF biomarkers of neuroinflammation were also reduced: YKL-40, IL-6, IL-1β and TNFα decreased by 9%, 14%, 11% and 5%, respectively. Plasma NfL, neurogranin, T-tau and YKL-40 levels were similarly reduced (Fig. 1). All reductions were of slightly lower magnitude in plasma except for neurogranin, which was reduced 40.7% in plasma. In contrast to the consistent and highly significant reductions of all other biomarkers, Aβ42 increased slightly in both CSF and plasma – a desirable result because low Aβ42 in CSF and plasma indicates AD. This increase, significant only in plasma due to variability in CSF, is consistent with PTI-125's mechanism: Aβ42 bound to α7nAChR is released due to a profound reduction in Aβ42's affinity for α7nAChR when PTI-125 binds altered FLNA and restores its native shape (2-4).

Figure 1. PTI-125 treatment reduces CSF and plasma biomarkers



All CSF biomarkers elevated in AD were significantly reduced in CSF after PTI-125 treatment. T-tau, NfL, neurogranin (Nrgrn) and YKL-40 were also significantly reduced in plasma. The slight increase in Aβ42 was significant in plasma but not in CSF due to variability. Inflammatory cytokines and P-tau in plasma were not assessed. *p < 0.0001, +p < 0.001, # p < 0.01 in paired t test comparing Day 28 to pre-dose baseline. N=12. Error bars are SEM.

Spaghetti plots of individual CSF biomarker values (pg/mL) show that each patient responded to PTI-125 treatment on virtually all biomarkers (Fig. 2). Interestingly, the two patients with high Aβ42 levels showed a decrease in this biomarker post-treatment. Familial AD mutations were not assessed but may have contributed. Although cytokines can be difficult to measure, the lower limits of quantitation (2x background)

JPAD - Volume 7, Number 4, 2020

were 3.9 pg/mL for IL-6 (R2 value 0.8864) and 7.8 pg/mL for both IL-1β and TNFα (R2 values 0.9374 and 0.8767, respectively).

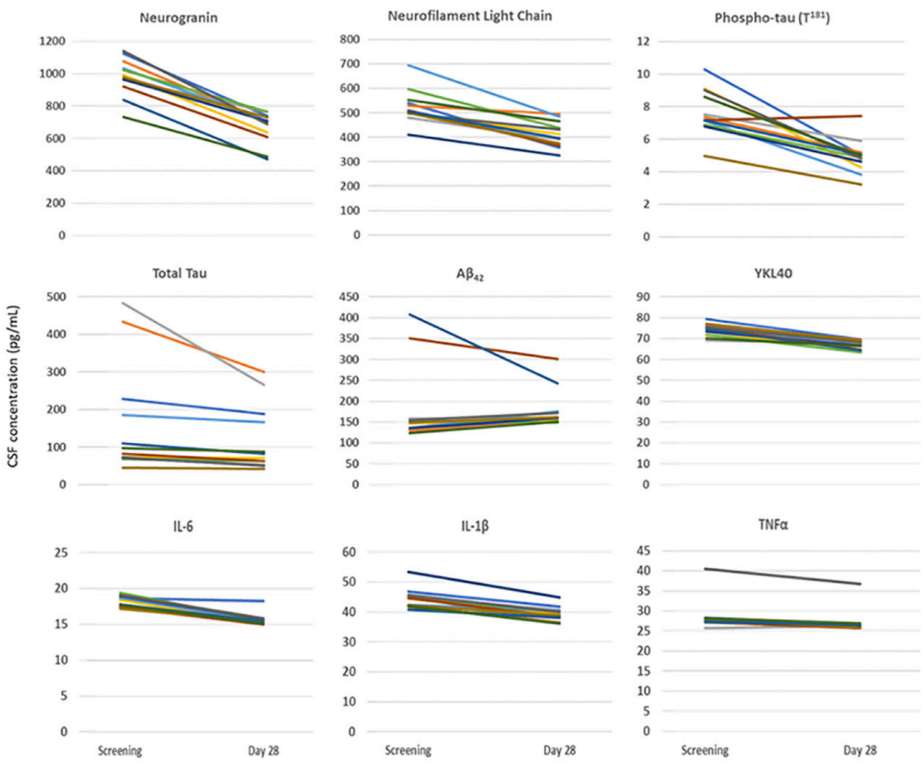
Because levels of phosphorylated tau defined by individual phospho-epitopes are low in CSF and even lower in plasma (as reflected in total tau levels), plasma levels of phosphorylated tau were assessed by immunoprecipitation of tau with anti-tau followed by immunoblotting of three different phospho-epitopes known to be elevated in AD. Tau phosphorylation at these sites (pT181-tau, pS202-tau and pT231-tau) was significantly reduced in plasma by 12.5%, 14.0% and 16.3%, respectively, following PTI-125 treatment (Fig. 3), corroborating pT181-tau results in CSF. Because tau is immunoprecipitated with a tau-specific antibody, tau levels serve as the control for phospho-tau and nitrated tau levels. The ratios for each P-tau epitope and nY to total tau were adjusted by the average reduction in total tau reduction (0.955 for Day 14 and 0.887 for Day 28; loading could not be adjusted in the experiment due to blinding). The higher than expected molecular weight for tau may be due to additional phosphorylation in blood; the anti-tau antibody used to detect tau is commonly used. The reduction in phosphorylated tau, together with 20.4% lower nitrated tau (nY29-tau; Fig. 3), suggests that PTI-125 can reduce tau hyperphosphorylation and oxidative stress to stabilize mitochondria and attenuate neurofibrillary lesions and neurodegeneration.

Mechanism and target engagement in patient lymphocytes

Patient lymphocytes, which express FLNA (24), α7nAChR and TLR4, were used to demonstrate target engagement and mechanism of action of PTI-125. Confirming the altered and acidic FLNA in AD brain (3), FLNA in patients' lymphocytes had an isoelectric focusing point (pI) of 5.3 prior to treatment. PTI-125 treatment reverted FLNA's pI from almost exclusively 5.3 on Day 0 to mostly 5.9 on Day 28 (Fig. 4a,b), indicating the reversal of pathological to physiological, native conformation (3).

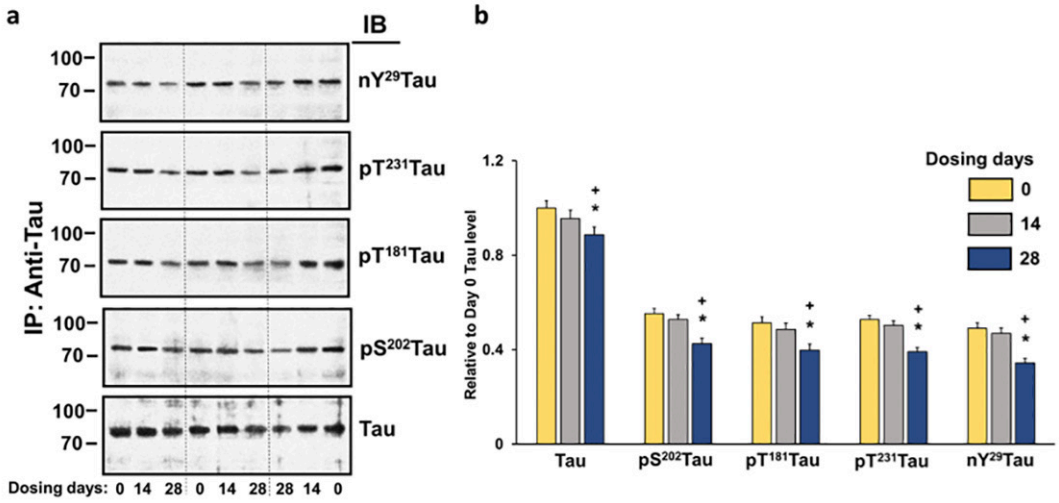
The benefit of this shift in FLNA conformation is shown by reduced FLNA linkages to α7nAChR and TLR4 in patient lymphocytes (by 45.4% for each, Fig. 4c,d) assessed by immunoblot detection of α7nAChR and TLR4 in anti-FLNA immunoprecipitates, as previously described for assessments in AD mouse models and AD postmortem human brain (3, 4). Finally, PTI-125 treatment benefit is also corroborated by reduced binding of Aβ42 to both α7nAChR and CD14, the co-receptor for TLR4, by 54.6% and 40.1%, respectively, demonstrated by immunoprecipitation with a specific anti-Aβ42 antibody and subsequent immunoblot detection of α7nAChR or CD14 in the immunoprecipitate (Fig. 5a, b). Immunoblot detection of Aβ42 itself in the immunoprecipitate showed

Figure 2. Individual patient changes in CSF biomarkers



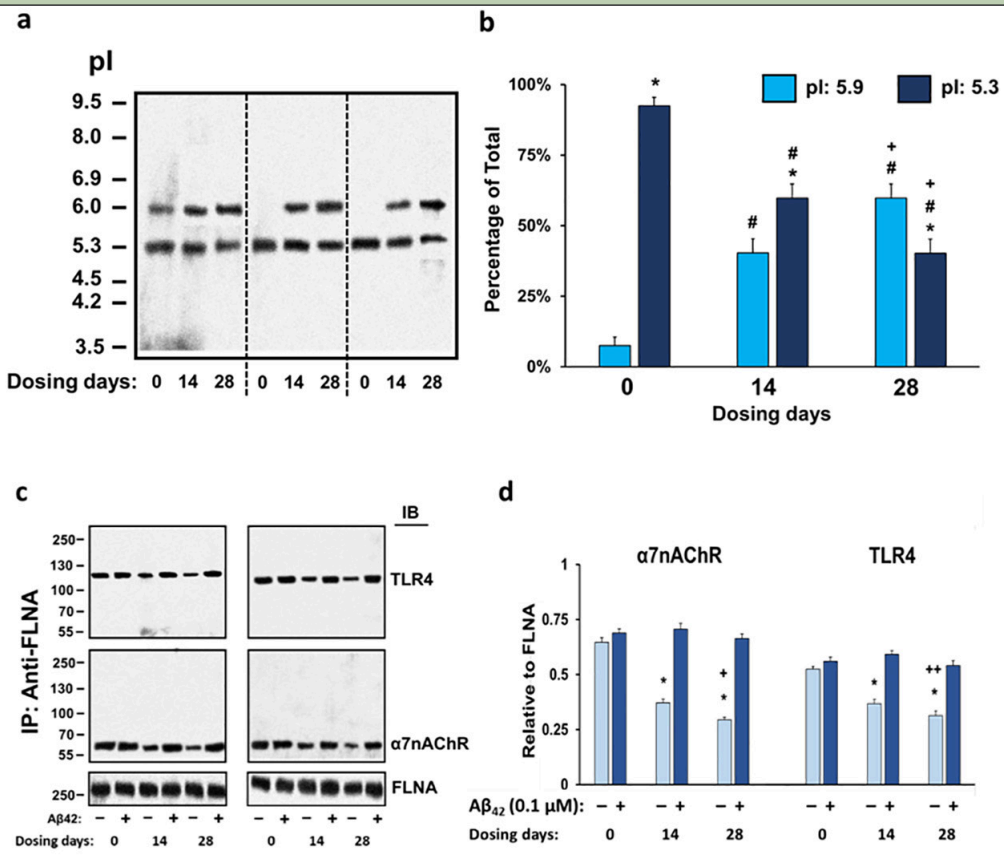
Each spaghetti plot shows reductions from baseline for each patient in one of nine biomarkers of Alzheimer's disease, neurodegeneration or neuroinflammation. Levels of Aβ42, usually low in AD patients, increased after treatment with PTI-125, except for two patients with high baseline Aβ42. Data are plotted as pg/mL.

Figure 3. PTI-125 treatment reduces phosphorylated and nitrated tau in plasma



a, b, Reductions in tau phosphorylation and tau nitration found in plasma following PTI-125 treatment. Reductions were evident by 14 days and stronger by 28 days of dosing, demonstrated by anti-tau antibody immunoprecipitation and immunoblotting with antibodies specific for each phosphorylation or nitration site. Blots (a) were evaluated by densitometric quantitation (b). *p < 0.001 vs. dosing day 0, +p < 0.01 vs. dosing day 14. N=13 with one missing value. Error bars are SEM.

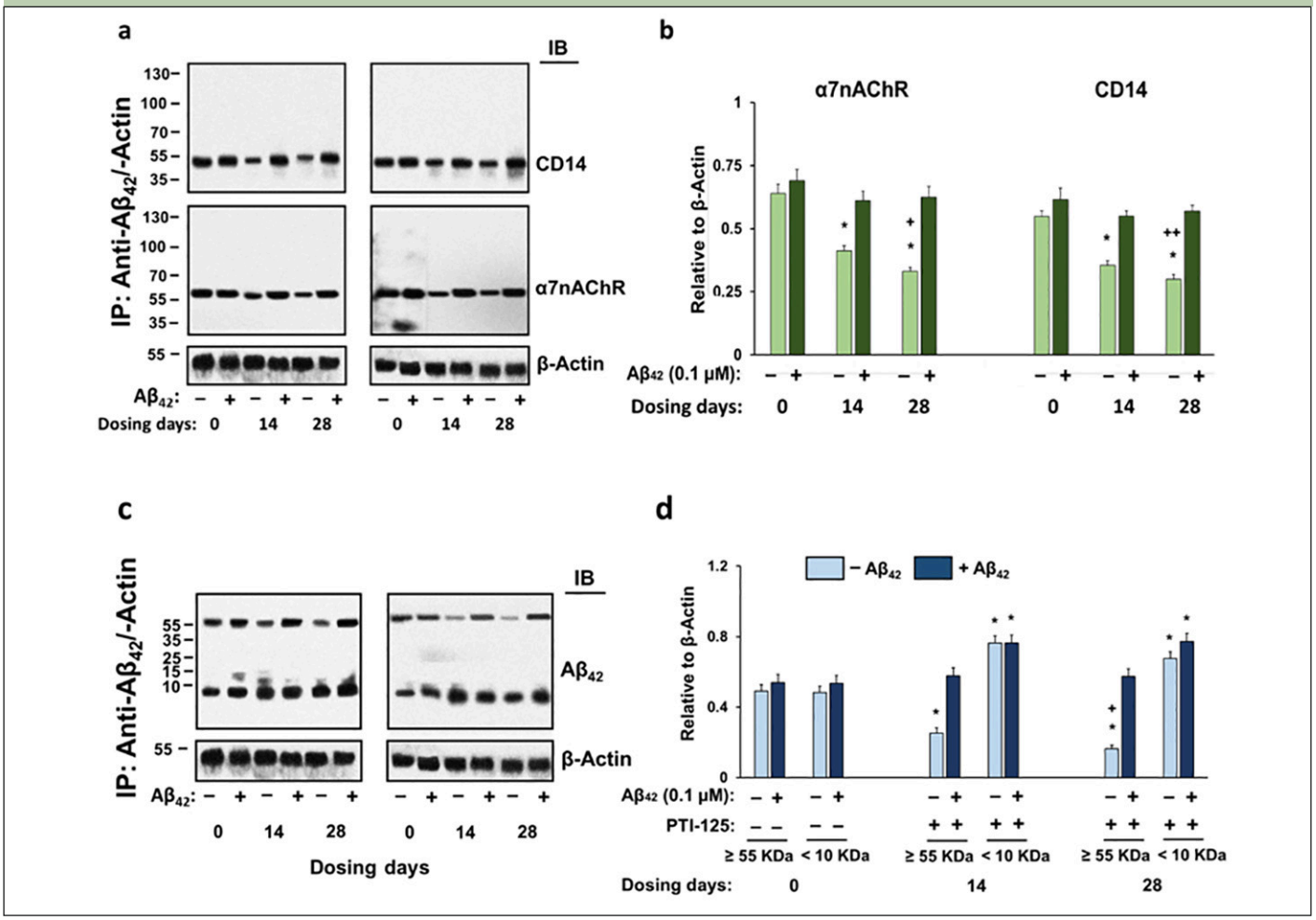
Figure 4. PTI-125 restores FLNA's native shape and reduces FLNA linkages to α7nAChR and TLR4



a, b Restoration of FLNA's native shape. Isoelectric focusing gel (a) and its quantitation (b) show that 93% of FLNA isolated from lymphocytes prior to treatment is in the altered conformation (pI 5.3), with just 7% in the native shape (pI 5.9). PTI-125 treatment for 28 days shifts this distribution to 40% in the altered and 60% in the native conformation. *p < 0.0001 comparing 5.9 to 5.3, #p < 0.0001 vs. dosing day 0, +p < 0.0001 vs. dosing day 14. c,d, Reductions in FLNA linkages to TLR4 and α7nAChR found in lymphocytes. Reductions are illustrated by TLR4 or α7nAChR levels detected using immunoblotting with specific antibodies in solubilized anti-FLNA antibody immunoprecipitates of lymphocytes. Additionally, exogenous Aβ42 added in vitro to lymphocytes reversed these reductions in FLNA associations, returning levels to pre-treatment baseline. Blots (c) were assessed by densitometric quantitation (d). *p < 0.001 vs. dosing day 0, +p < 0.01 and ++p < 0.05 vs. dosing day 14. N=13 with one missing value. Error bars are SEM.

JPAD - Volume 7, Number 4, 2020

Figure 5. PTI-125 treatment reduces levels of Aβ42 bound to α7nAChR and CD14



a, b, Reductions in levels of complexes of Aβ42 with α7nAChR and CD14 found in lymphocytes. Reductions are illustrated by α7nAChR and CD14 levels detected using immunoblotting with specific antibodies in anti-Aβ42 antibody immunoprecipitates of lymphocytes. c, d, The 57-KDa band representing Aβ42 tightly bound to CD14 or α7nAChR is progressively reduced over dosing days. Aβ42 in lymphocytes (endogenous or exogenously added) is predominantly monomeric, illustrated by size < 10 KDa (c). As with FLNA linkages, exogenous Aβ42 added in vitro to lymphocytes reversed these reductions in Aβ42 – α7nAChR/CD14 complexes, returning levels to pre-treatment baseline. Actin simultaneously immunoprecipitated by anti-actin antibodies was used as a loading control for both blots. Blots (a, c) were assessed by densitometric quantitation (b, d). *p < 0.001 vs. dosing day 0, +p < 0.01 and ++p < 0.05 vs. dosing day 14. N=13 with one missing value. Error bars are SEM.

a <10 KDa species that increases following treatment, indicating small oligomers or monomers, as well as a band of 57 KDa, representing Aβ42 monomers tightly bound to α7nAChR or CD14 (7, 25), which decreased with treatment (Fig. 5c, d). The reduction in Aβ42 bound to α7nAChR is consistent with PTI-125's 1000-10,000-fold reduction in binding affinity of Aβ42 to α7nAChR (4).

The fact that reduced FLNA linkages and Aβ42 binding to α7nAChR/CD14 can be reversed by adding exogenous Aβ42 (0.1 μM) illustrates the dynamic nature of this Aβ42-mediated pathogenesis in AD. Because CSF biomarkers were notably reduced, these findings in lymphocytes of PTI-125-treated patients can be inferred also to occur in brain. In support, reductions in FLNA linkages to α7nAChR and TLR4 in lymphocytes (unpublished data) of transgenic AD mice treated with PTI-125 tracked similar reductions we reported in brains of these mice (3).

Discussion/Future development plans

In a first-in-patient clinical trial of PTI-125, CSF and plasma biomarkers of AD pathology, neurodegeneration and neuroinflammation were markedly improved following 28-day oral treatment with PTI-125. All patients showed a biomarker response to PTI-125. The drug was well tolerated, with no observable drug-related adverse events. PTI-125's effects in patients are consistent with its mechanism of action and published preclinical data. Target engagement and mechanism of action of PTI-125 were demonstrated in patient lymphocytes by reduced associations of FLNA with α7nAChR and TLR4, reduced binding of Aβ42 to α7nAChR or CD14 and a shift back to FLNA's native shape, visible by isoelectric focusing point. The magnitude and consistency of reductions in several established, objective biomarkers following treatment with PTI-125 at 100 mg twice daily for 28 days imply a slower rate of neurodegeneration or a suppression of

disease processes in AD.

Cognition and function were not assessed in this small study. However, elevated CSF biomarkers of P-tau and total tau/Aβ42 ratio have previously been correlated with worse performance on a wide range of memory and sustained attention assessments (26) and define the disease state if not also progression. We therefore hypothesize that decreasing these markers will favorably impact patient cognition and function or their rates of decline. Additionally, other research has shown that 11-13% decreases in CSF neurogranin and P-tau and a slower increase in CSF NfL compared to placebo over 18 months is associated with a slower rate of cognitive decline in prodromal AD patients (27).

Based on these encouraging safety and biomarker data, patients are currently being enrolled in a Phase 2b study to assess the safety, tolerability and effects of PTI-125 in patients with mild-to-moderate AD. This blinded, randomized, placebo-controlled, clinical trial will enroll approximately 60 patients with mild-to-moderate Alzheimer's disease. In the Phase 2b study, patients are administered PTI-125 100 mg, 50 mg or matching placebo, twice daily, for 28 continuous days. The primary endpoint is improvement in biomarkers of Alzheimer's disease from baseline to Day 28. Although Phase 2b is too small (N=60) to generate statistically meaningful data in cognition, a cognition scale (beyond MMSE) is being utilized to guide statistical considerations for future, large-scale clinical investigations with PTI-125. Unambiguous improvements in cognition and function is a key efficacy criterion for FDA approval of a new drug in AD (28), a hurdle which, to date, no drug candidate for AD has met with clear and compelling clinical data. Ultimately, to demonstrate disease modification in AD, future investigations must correlate improvements in biomarkers by PTI-125 with beneficial effects on cognition and function.

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Conflict of interest: LHB, CAC, RB and NF are employees of Cassava Sciences, Inc. HYW and MRM are science advisors to Cassava Sciences, Inc. ELB and BN are employees of IMIC, Inc., an independent clinical site that participated in this study.

Role of the sponsor: Cassava Sciences, Inc. provided all drug supply and material support for this clinical research, designed the study in consultation with its advisors and monitored the conduct of the study and data collection. Biomarker assays were conducted blind to treatment day by HWY and his lab at CUNY Medical school. LHB assisted in the interpretation of the data and wrote the manuscript together with RB and HWY.

Ethical standards: All participants and their caregivers provided written informed consent. The protocol, informed consent forms and clinical sites were all approved by Advarra IRB.

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